DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
Rockville MD 20857

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FAP 2234

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Bruce D. King, Ph. D.
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DuCoa
P. O. Box 219
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Dear Dr. King:

On June 30, 1995, you submitted a food additive petition in which your company seeks to amend Title 21, Part 573 of the Code of Federal Regulations (21 CFR 573) to permit the use of natamycin, at a level of 11 ppm, as a "mold retardant of Asperigillus parasiticus, Penicillium rubrum, and Fusarium moniliforme for up to 14 days in broiler chicken feed." You have amended the petition three times; first on July 17, 1995, and then on August 18, 1995, and October 26, 1995.

The amended petition was reviewed as follows:

Chemical Identity - Manufacturing Controls and Chemistry

The manufacturing controls and chemistry part of the petition is found to be satisfactory. The method of analysis for natamycin used in production quality control and product release, stability studies of the premix and treated feeds, is correct, useful and under statistical control. The natamycin premix "Nsure" is stable to one year and treated feeds should provide the requisite amount of mold retardant to at least four weeks. The product packaging should bear the expiration date of one year from date of manufacture.

Utility

You conducted sixteen experiments, in the laboratory and under actual conditions of use, to establish the utility of natamycin for retarding the growth of Aspergillus parasiticus, Fusarium moniliforme, and Penicillium rubrum. Detailed reports of the experiments were provided in volumes 6, 7, 8, 9, 10, 33, and 34 of your petition. The reports included an extensive preamble in which you discussed the significance of molds in agriculture, described weaknesses in the traditional methods of analyzing molds, and introduced a new respirometer (micro-oxymax 20) that you claim to be capable of overcoming those weaknesses. The micro-oxymax 20 was modified by you to enable the simultaneous measurement of changes that occur in levels of oxygen (O_2) and carbon dioxide (CO_2) during the growth of different molds.

Laboratory Studies

Twelve experiments were conducted in the laboratory. One was conducted to demonstrate weaknesses in one of the traditional methods of analyzing molds, three to demonstrate the ability of the respirometer to measure mold growth, seven to determine the minimum dose of natamycin that is effective in retarding the growth of Aspergillus parasiticus, Penicillium rubrum and Fusarium moniliforme, and another one to confirm the ability of the selected dose to retard the growth of the specified species of mold.

Dose determination studies

The first six and twelfth experiments reported were conducted to determine the minimum dose of natamycin that is effective in retarding the growth of Aspergillus parasiticus, Penicillium rubrum and Fusarium moniliforme. The respirometer was used in the first five experiments while the sixth and the twelfth relied on a traditional method of analysis, or a combination of respirometry and traditional methods, respectively.

The quality of the experiments conducted to determine the minimum effective dose of a substance is pivotal to the success of a food additive petition for that substance. The experiments you conducted for this purpose for natamycin were generally well designed and would have permitted an independent evaluation of the ability of natamycin to retard the growth of the specified molds if it were not for the following deficiencies:

You did not provide evidence to show that the autoclave procedure was effective in sterilizing the flasks, solutions, feeds and other materials used. Because microorganisms other than the targeted species of mold can also consume O_2 or produce CO_2, and the number of those microbes can vary between experimental and control groups, it is essential that all extraneous microbes should be eliminated from all materials before inoculation of materials with the targeted species of molds. That should ensure that differences observed between the levels of O_2 and CO_2 consumed or produced in the experimental and control groups can be attributed only to differences in activities of the molds being tested. The sterilization of materials used is one important tool for achieving this purpose and you are requested to provide evidence that sterilization was achieved by the autoclave procedure used. The evidence should be provided in the form of results of microbiological analyses conducted before and after autoclaving. The analyses, should at the minimum, include total aerobic counts and tests for the presence or absence of molds.

The experiments should also have included negative controls consisting of chambers containing only sterilized feed or, in the case of the 12th experiment, the original, non-hydrated, non-treated feed.

To permit an independent evaluation of data submitted, it is necessary for you to provide us with the following information:

(a) Moisture content; the moisture contents of feed samples as determined by the AOAC method, the amounts of water added to each feed to readjust its moisture level, and the results of the analyses conducted to determine the new moisture levels.

(b) Spore concentration; the concentration of mold spores in the suspensions used for inoculation, as determined by the standard dilution and pour plate technique, and the volume of suspension used each time.

(c) Natamycin concentration; the specific amounts of natamycin premix added to feeds to yield groups of feed containing the various concentrations of natamycin tested. The feeds should also have been analyzed to determine the quantity of natamycin actually present in each group.

Please explain the need for pre-incubation at 30 °C before the chambers were connected to the respirometer, and why the length of pre-incubation varied so widely between experiments.

The four or five 20 g samples extracted from each 200 g aliquot of feed already treated with mold spores and natamycin are not replicates as you claim. The experiments need to include the use of two or more real replicates for mold spore inoculation and natamycin addition.

Concerning your explanation of the increases in O_2 and CO_2 observed in the first experiment with blank culture chambers, another interpretation of the results is possible as follows: since no evidence was provided to show that the autoclave procedure sterilized the culture flasks or "humidifiers", it is possible that the increases observed in the amounts of O_2 and CO_2 consumed or produced, respectively, were caused by microbes that survived the autoclave procedure.

Several other aspects of the results of these experiments also give rise to some concerns. The graphs used to summarize the results were quite confusing and did not bear the titles referred to in the texts. Although attempts were later made to produce clearer graphs, you did not indicate whether or not the newer graphs were to be used as replacements for the original graphs. Moreover, the newer graphs were located in a volume of the submission that was totally separate from that containing the original graphs. The raw data for the second experiment was located in the submission after those for the third experiment, and contained no information about cumulative O_2 consumption or CO_2 production even though the text relied heavily on those parameters. Please explain how the cumulative values referred to in the text, tables, and graphs in this and the third experiments, were derived. You are also requested to explain the discrepancies between printed and hand-written information contained in the raw data for the second experiment. An example of the discrepancies is the printed statement that the experiment was started on 1/1/80, whereas a hand-written note states that inoculation occurred at 4:45 pm on 11/10/89. Finally, for the fourth, fifth, and twelfth experiments, we will appreciate explanations for the discrepancies that exist between the contents of texts and raw data. Examples of the discrepancies include statements in the

texts that cultures were pre-incubated for 40 and 115 hours in the fourth and fifth experiments, respectively, whereas the corresponding information in the raw data specifies "no significant pre-incubation" for the fourth experiment and "60 hours of pre-incubation" for the fifth. Also, the text for the fifth experiment claims a spore concentration of 5.0×10^3/g while the raw data specifies 7.0×10^3/g. Moreover, the text of the twelfth experiment says respirometric sampling was conducted every 4 hours whereas the raw data specify measurements at 6-hour intervals. Most of the raw data submitted for the fourth experiment were not legible.

The results of the fifth experiment indicate either some problem with the respirometer or a lack of effectiveness of natamycin against Penicillium rubrum. You speculated that the poor result was attributable to the slow growth rate of Penicillium rubrum in feed at the moisture level used. If that is correct, and you still intend to make a claim for the effectiveness of natamycin against Penicillium rubrum, you are requested to repeat the experiment under conditions demonstrated by you to be the optimum for supporting the normal growth of the mold.

The concerns raised by the sixth experiment include the total lack of information about the scoring team: What was its composition, and the training or qualifications of its member(s)? Was the team "blinded" to the experimental design? Also, although each of the scores presented in the Table was said to be the average of four values, you did not provide the variation around those averages or the raw data that would have permitted an independent estimation of those variations.

One major concern raised by the report of the twelfth experiment is the fact that a letter included in the report, and said to have been written by the director of the laboratory where samples were analyzed by scanning electron microscopy (SEM), stated that the samples for SEM were received by the laboratory on July 11, 1991 - the date specified in the raw data as that on which the experiment itself was started. Also, the letter clearly shows that the director was not "blinded" to the design of the study. Moreover, an effort should have been made to determine if the three targeted species of mold were present in the feed used. Their presence would have made the experiment more pertinent and useful in establishing the utility of natamycin for the proposed use.

With regard to the statistical procedures used in analyzing the results of these dose determination studies, we do not recommend sole reliance on the use of tests comparing means of treatment groups. Our preferred method of determining the minimum effective dose is modeling the dose response curve. Accordingly, we tried to determine the best model by fitting various models to the oxygen consumption data provided. Simple linear regression turned out to be one of the top choices and we used it in our analysis of your data. Based on that analysis, and assuming the data are reliable, we are recommending a minimum effective dose of 15 g/ton (16.6 ppm) for natamycin, instead of the 10 g/ton (11 ppm) that you propose. A copy of the results of our review of the statistical sections of your utility studies is enclosed for your information.

Respirometric measurements and mold growth

The seventh, eighth, and ninth experiments were conducted to demonstrate the existence of a strong positive correlation between the growth rate of a mold as measured by traditional methods and changes in the cumulative amounts of O_2 consumed or CO_2 produced over time by the mold, as measured by the micro-oxymax 20 respirometer. The mold tested in each was Aspergillus parasiticus, and the traditional method used to measure growth rate was the determination of changes in the weight of mycelia over time.

It was essential for you to conduct these experiments, because your company was using respirometry to establish its claim that natamycin can retard the growth of the three targeted species of molds. Since changes in the amounts of O_2 and CO_2 measured using a respirometer can only be regarded as an indication of changes in the metabolic activity of the molds, and since growth is only one of several possible outcomes of metabolic activity, it was necessary for you to show a direct correlation between growth of the molds and the O_2 and CO_2 consumed or produced, respectively, during that growth.

The seventh experiment alone would have sufficed in achieving the purpose outlined above. However, its design was deficient in one important respect: all the flasks (instead of only four) should have been connected to the respirometer, and a set number of them disconnected from the respirometer at specified intervals and their contents filtered and weighed as described. This would have enabled a more valid correlation of mycelial weight and O_2 and CO_2 measurements. Other important deficiencies include the fact that the raw data presented in support of the seventh experiment were not compatible with the design and results of the experiment as described, and a materials and method section and raw data were not presented for the eighth and ninth experiments, respectively. Also missing were several pertinent pieces of information (proof of sterility, quantity of materials used, etc.) that would permit an independent evaluation of the data presented. We suggest that you should consider conducting a new experiment to address these concerns.

Other Laboratory Experiments

The tenth experiment was conducted to demonstrate that the respirometry was a more dependable methodology than mold spore count for measuring mold growth. The results were said to indicate no significant changes in the concentration of mold spores throughout the duration of the experiment, whereas there were steady increases in the quantities of O_2 and CO_2 consumed or produced, respectively. Since mold spores develop into the molds that consume or produce the O_2 or CO_2, it is difficult to comprehend the lack of change in the concentration of mold spores throughout the duration of the tenth experiment while there were steady increases in the quantity of O_2 and CO_2. The problem might be due to deficiencies in experimental design including the inoculation of excessive amounts of mold spores.

The eleventh laboratory experiment was conducted to use respirometry to confirm the ability of natamycin (11 ppm) to retard the growth of the three species of mold targeted by your petition. The challenge inoculum used consisted of a 1:1:1 ratio of spores of Aspergillus parasiticus, NRRL 2999, Fusarium moniliforme, NRRL 5806, and Penicillium rubrum, NRRL 3290. The final concentration of spores in the experimental feed was said to be 3.3×10^4 spores/g. After the termination of respirometry, you cultured samples of the control and natamycin-treated feeds to determine the predominant species of mold present.

You claimed that your results showed that, starting on day six of respirometry and continuing till the end at 14 days post-inoculation, the cumulative amounts of O_2 consumed and CO_2 produced were significantly ($p<0.05$) less in the natamycin-treated group than in the control. It was also stated that cultures of the contents of flasks from both groups established Penicillium rubrum as the predominant mold species. It was impossible to conduct an independent evaluation of the experiment because your report of the experiment contains several deficiencies. The deficiencies are quite similar to those noted earlier for other experiments (especially the first six), and include the absence of raw data and other important pieces of information.

Field trials

Four experiments were conducted to confirm the ability of natamycin (11 ppm) to retard the growth of the three targeted species of mold in broiler chicken feed under actual conditions of use. One experiment each was conducted in Maryland and Louisiana, and two in Georgia. The design of the experiments was identical. However, the second experiment in Georgia involved the use of a considerably larger number of broiler chicken farms.

The experiments were well designed, included adequate controls and would permit an independent evaluation of the confirmation of the ability of natamycin (11 ppm) to retard the growth of the three targeted species of mold in broiler chicken feed under actual conditions of use. However, as indicated by the results of your own analysis of the data obtained (there were numerical, but no statistically significant, differences between groups) the tested dose failed to achieve the intended effect. The failure could be attributed to several factors including the fact that the amounts of natamycin actually present in the feeds (average of 8 ppm) were much lower than the 11 ppm intended. It is also possible that the proposed dose of 11 ppm is too low, and that the minimum effective dose is 15 g/ton (16.5 ppm) as indicated by our analysis of the data from your laboratory experiments. Your response to the concerns we have about the laboratory experiments should help to clarify the issue.

Proposed purposes and amounts, proposed label, and proposed regulations

Under the proposed purpose section of your submission, you petitioned that regulations should be amended to permit the use of natamycin premix at the rate of one pound per ton for the purpose of retarding the growth of Aspergillus parasiticus, Penicillium rubrum, and Fusarium moniliforme in broiler chicken feed for up to 14 days. However, in the section on proposed regulation your petition seeks to permit the use of natamycin as a mold retardant for the specified molds. A copy of your proposed label was enclosed in your submission.

Because the proposed purposes and amounts, and important portions of the proposed label and regulations will depend heavily on the outcome of the section on utility, we are reserving full comments on the three sections until after the utility of natamycin for its intended purpose has been satisfactorily established. However, at this stage, we wish to state our preference for the description ("retarding the growth of ... ") specified in your proposed purpose section, over that ("as a mold retardant of ...") used in your section on proposed regulation. In addition to specifying the active ingredient, natamycin, it will be necessary to list all the ingredients present in "NSURE." Also, we request that you should consider developing and including language that will enable users of "Nsure" to avoid the type of problem observed in the field trials when the product was added to broiler feed at the same time as liquid feed components. Moreover, because the actual concentrations of natamycin in feeds used in the field trials were uniformly lower than the intended concentration and those lower levels did not appear to be effective, we suggest that you develop a mixing technique that will ensure that the amount of natamycin actually present in treated feed is close to that intended. A description of that technique should also be included in the directions for use.

Human Safety

We find this section of your submission to be satisfactory and have no human food safety concern at this time regarding the use of natamycin at 11 ppm in broiler chicken feed. However, please note the following:

1. Using a No Observed Effect Level (NOEL) of 50 mg/kg bw/day obtained from studies previously reviewed by us and summarized for you in our letter dated January 14, 1992, and a 1000-fold safety factor, an acceptable daily intake (ADI) of 50 µg/kg body weight/day has been calculated as the safe concentration (SC) for total residues of natamycin. Because of limitations set forth in the guidelines concerning antimicrobial drug residues, the ADI for natamycin cannot exceed 25 µg/kg bw/day (1.5 mg/person/day).

2. Since the limitation in ADI arises from concerns with microbiological residues, a higher ADI could be assigned (up to 50 µg/kg bw/day) if microbiological studies were done to demonstrate that the higher ADI had no adverse effects on intestinal microflora. Alternately, residue depletion studies could be performed in food animals to demonstrate that the microbiologically active portion of the total residues in a given food animal does not exceed 25 µg/kg bw/day.

3. Calculation of SC from the ADI
In cases where drugs will enter either the milk or egg supply, a portion of the ADI is set aside for such use. Since it is our understanding that natamycin may be used in laying hens, a portion of the ADI must be set aside for potential residues in eggs.

a) ADI for muscle = Total ADI (25 µg/kg bw/day) - 20% set aside for eggs

$$SC_{muscle} = 0.8 \times 25\ \mu g/kg\ bw/day \times \frac{60\ kg\ (\text{weight of average person})}{0.3\ kg\ (\text{muscle consumption/day})} = 4\ ppm$$

b) Set aside for eggs = 20% of ADI

$$SC_{eggs} = 0.2 \times 25\ \mu g/kg\ bw/day \times \frac{60\ kg\ (\text{weight of average person})}{0.1\ kg\ (\text{egg consumption/day})} = 3\ ppm$$

c) The SC in liver, kidney and fat are based on the daily consumption levels of 100 gms, 50 gms and 50 gms, respectively, and are:

SC_{liver}	12 ppm
SC_{kidney}	24 ppm
SC_{fat}	24 ppm

4. We have used a 20% set aside for potential residues of natamycin in eggs solely as a reference point. You may wish to consult with us on the set aside as well as the microbiological limitations we have used.

Target Animal Safety

We find this section of your petition to be satisfactory.

Environmental Assessment

We have carefully considered the potential environmental effects associated with the approval of natamycin for use as proposed, and determined that the manufacture and use of the product is not expected to have a significant impact on the human environment and that an environmental impact statement is not required. Therefore, we have prepared a finding of no significant impact (FONSI) for this action.

In conclusion, we have reviewed your recent food additive petition for natamycin and found the sections on chemical identity - manufacturing controls and chemistry, human safety, and environmental assessment to be satisfactory. However, the sections on utility, proposed purposes and amounts, proposed regulations, and the proposed label are incomplete.

You can either amend your petition, as provided under 21 CFR 571.6, by submitting additional data to address the concerns we have expressed in this letter, or withdraw the petition as provided for in 21 CFR 571.7.

Please do not hesitate to contact us if you have any questions about the contents of this letter. Our telephone number is (301)-594-1731.

Sincerely yours,

George Graber

George Graber, Ph.D.
Director
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Enclosure